# Inhibition of Interferon-γ–Induced Intercellular Adhesion Molecule-1 Expression on Human Keratinocytes by Phosphorothioate Antisense Oligodeoxynucleotides Is the Consequence of Antisense-Specific and Antisense–Non-Specific Effects

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**Expression of intercellular adhesion molecule-1 (ICAM-1) by keratinocytes is an important event in the pathogenesis of T-cell–mediated inflammatory skin diseases. To determine if ICAM-1 expression could be selectively modulated, two antisense phosphorothioate oligonucleotides (S-ODN) targeting the translation initiation and 3′ untranslated regions of ICAM-1 mRNA were added as lipid complexes to cultures of keratinocytes. Interferon-γ was added after 24 h to induce ICAM-1 expression, which was quantitated by flow cytometry after 48 h. The S-ODN targeting the translation initiation site did not inhibit ICAM-1 expression at 0.2–20.0 μM. In contrast, 0.2–1.0 μM of the S-ODN targeting a site in the 3′ untranslated region abrogated ICAM-1 expression in up to 75% of the keratinocytes; this inhibition was reversible when complementary sense S-ODN was added. Phosphodiester ODN (PD-ODN) targeting the same sites did not inhibit ICAM-1 expression on keratinocytes, most likely as a consequence of rapid degradation. Inhibition of ICAM-1 by the antisense S-ODN was selective; expression of β2-microglobulin, α3-integrin, and β1-integrin remained largely unaffected and interferon-γ–induced HLA-DR expression was inhibited to a much lesser extent than ICAM-1. Antisense–non-specific inhibition was also noted in that two scrambled S-ODN with an identical nucleotide (14 of 20 cytosines) composition inhibited ICAM-1 expression in up to 44% of the keratinocytes, whereas a degenerate S-ODN did not. The data demonstrate the complex effects exerted by antisense S-ODN in that ICAM-1 expression was inhibited via antisense–non-specific mechanisms probably due to the intrinsic properties of the S-ODN as well as via the anticipated sequence-specific mechanisms.** *J Invest Dermatol 104:813–818, 1995*

Antisense technology has gained increasing experimental and clinical interest as a tool to selectively modulate expression of particular gene products [1–5]. Antisense oligodeoxynucleotides (ODN) have been demonstrated to block *in vitro* translation of mRNAs in a sequence-specific manner. DNA oligomers present distinct advantages over RNA oligomers in that they are more stable in cell-free systems and in cultured cells [1,6]. Because the utility of ODN with the conventional phosphodiester backbone structure is limited due to their high susceptibility to degradation by nucleases, modified ODN such as phosphorothioates (S-ODN) have been developed and successfully employed [7–8]. Some striking examples for the *in vitro* effects exerted by S-ODN are the inhibition of HIV replication [9], oncogene expression [10,11] and modulation of cytokines and growth factors [10,12–14].

The aim of this study was to determine if interferon-γ (IFN-γ)–induced intercellular adhesion molecule-1 (ICAM-1) expression on cultured human keratinocytes could be inhibited by phosphorothioate ODN (S-ODN). ICAM-1 is upregulated on keratinocytes in various inflammatory skin disorders and is thought to be a critical factor in lymphocyte trafficking [15]. Inhibition of ICAM-1 expression on keratinocytes may be beneficial in the treatment of T cell–mediated inflammatory skin diseases. We tested two antisense S-ODN; one targeting the translation-initiation site and the other a stem-loop structure at the 3′-untranslated region of ICAM-1 mRNA that have been previously identified as inhibitors of cytokine-induced ICAM-1 expression [16]. We have also characterized parameters that are critical for antisense-induced ICAM-1 inhibition in keratinocytes, such as the presence of liposomes and the backbone nature of the antisense ODN.

## MATERIALS AND METHODS

**Reagents** Lipofectin was purchased from Gibco BRL (Gaithersburg, MD) and used at 10 μg/ml in all experiments. Recombinant human IFN-γ was obtained from Boehringer Mannheim (Inglewood, NJ). Fluorescein

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Abbreviations: AS, antisense; DEG AS, degenerate di-mer antisense; PD-ODN, phosphodiester oligodeoxynucleotides; S-ODN, phosphorothioate oligodeoxynucleotides.

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**Table I. Oligonucleotides**

| ODN | Sequence (5′..3′) | Specificity | Backbone |
|---|---|---|---|
| AS1 | CCC CCA CCA CTT CCC CTC TC | 3′-untranslated region (ISIS 1939 in [16]) | S-ODN |
| PD-AS1 | CCC CCA CCA CTT CCC CTC TC | 3′-untranslated region (ISIS 1939 in [16]) | PD-ODN |
| SCR1a | CCT CAC CCC CCT ACC TCT CC | Scrambled to AS1 | S-ODN |
| PD-SCR1a | CCT CAC CCC CCT ACC TCT CC | Scrambled to PD-AS1 | PD-ODN |
| SCR1b | TCC ACC CTC CAC TCC CTC CC | Scrambled to AS1 | S-ODN |
| SS1 | GAG AGG GGA AGT GGT GGG GG | Sense strand to AS1 | S-ODN |
| AS2 | TGG GAG CCA TAG CGA GGC | AUG translation initiation codon (ISIS 1570 in [16]) | S-ODN |
| SCR2 | GCA TAG GGC GAA CGG TGC | Scrambled to AS2 | S-ODN |
| DEG AS | 21-mer | Degenerate | S-ODN |

isothiocyanate (FITC)–conjugated $F(ab')_2$ fragments of goat anti-mouse IgG were obtained from Tago, Burlingame, CA. Mouse IgG2a and a monoclonal antibody (MoAb) to HLA-DR (mouse IgG2a) were purchased from Becton Dickinson (San Jose, CA), MoAb (mouse IgG2a) to ICAM-1 (CD54) and β2-microglobulin from Monosan (Uden, Netherlands). MoAb from mouse ascites against β1- and α3-integrin were purchased from Chemicon (Temecula, CA).

**Oligonucleotide Synthesis** Phosphorothioate (S-ODN) and phosphodiester (PD-ODN) oligodeoxynucleotides complementary to two regions of the human ICAM-1 mRNA that had been previously identified as sensitive targets for antisense-induced inhibition [16] were synthesized by cyanoethyl phosphoramidite chemistry on an Applied Biosystems Model 380B DNA synthesizer. Sulfurizing reagent (Glen Research, Sterling, VA) was used according to the manufacturers' instructions. ODN were ethanol-precipitated in the presence of sodium acetate, washed in 70% ethanol, and resuspended in sterile water. Aliquots of each sample were analyzed for purity by acrylamide denaturing electrophoresis. The concentration of the synthesized ODN was determined by their optical density at 260 nm. The characteristics and sequences of the ODN employed are listed in **Table I**. Additionally, phosphorothioate trinucleotides (G-G-G, G-A-G, T-G-G, G-T-G, G-T-G, G-G-A) complementary to nucleotide sequences shared by AS1, SCR1a, and SCR1b were also synthesized. Internally fluorescinated S-ODN (FITC-ODN) were synthesized using fluorescein-on phosphoramidite (Clontech, Palo Alto, CA) following manufacturer's instructions.

**Human Keratinocytes** Normal human epidermal keratinocytes were purchased from Clonetics (San Diego, CA) and grown in a modified serum-free keratinocyte growth medium (Gibco). All keratinocytes were utilized in experiments at the fourth to sixth passages. Cells were cultured in a humidified incubator at 5% $CO_2$ and 37°C.

**Oligonucleotide Treatment of Keratinocytes** $10^6$ keratinocytes were cultured in 25-ml culture flasks (Falcon, Oxnard, CA) overnight to allow adherence. The next day, culture media were removed and cells were incubated in medium (2 ml) containing lipid-ODN complexes at 37°C for 4 h. Lipid-ODN complexes were formed by incubating 10 μg/ml lipofectin with different concentrations of ODN added as 100× stock solutions for 15 min at room temperature prior to addition to cells. After the 4-h incubation period, flasks were twice washed with 5 ml $Ca^{++}$- and $Mg^{++}$-free phosphate-buffered saline (PBS) (Biofluids, Rockville, MD) and fresh medium supplemented with S-ODN, but without lipofectin, was added. Cells were incubated for an additional 24 h at 37°C and then stimulated with 1000 U/ml recombinant human IFN-γ (Boehringer) for an additional 24 h (previously determined to be optimal for inducing ICAM-1 expression).

**Phenotypic Analysis of Cells by Flow Cytometry** Keratinocytes were removed from tissue-culture flasks by incubation at 37°C in a 0.25% trypsin/ethylenediaminetetraacetic acid solution (Gibco) for 2–4 min. For flow-cytometry studies, cells were washed twice in PBS supplemented with 5% fetal bovine serum and 0.02% $NaN_3$, the buffer used in all subsequent washes. $10^6$ cells were incubated with 15 μl of unconjugated specific or control antibody (mouse IgG2a) at 10–15 μg/ml at 4°C for 30 min. After three washes, cells were incubated with 15 μl of a fluoresceinated goat anti-mouse antibody (1:1000). Samples of $10^4$ viable cells were then analyzed by flow cytometry on a FACScan II (Becton Dickinson) using the consort 30 software program. Dead cells were excluded by gating out cells containing propidium iodide (50 μg/ml in PBS).

**Immunofluorescence Microscopy** Keratinocytes ($10^6$) were seeded in chamber slides (Nunc, Naperville, IL) 24 h prior to the standard antisense treatment (as described above). After treatment with fluoresceinated (FITC) antisense ODN for 24 h, the chambers were removed and the slides were washed three times in PBS. Slides were air-dried and were mounted immediately prior to fluorescence microscopy to prevent elution of FITC-ODN from the cells.

## RESULTS

**Effect of Lipofectin on IFN-γ–Induced Upregulation of Keratinocyte Surface Molecules** Keratinocytes do not constitutively express ICAM-1 and HLA-DR on their surface [17]. For this reason, keratinocytes were incubated (24 h) with 1000 U/ml of IFN-γ, a known inducer of these surface molecules [17]. Because preliminary experiments showed that ICAM-1 AS1 and AS2 did not affect ICAM-1 expression on keratinocytes at concentrations of up to 5 μM (data not shown), we attempted to increase the cellular uptake of the ODN as a complex with the cationic lipid lipofectin, as previously reported [16,18]. In preliminary experiments, the effect of different concentrations of lipofectin (0.1–20.0 μg/ml) on the cellular metabolism of keratinocytes was studied using the MTT [3, (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. A concentration of 10 μg/ml lipofectin, added to cultures of keratinocytes for 4 h, was chosen because it was the highest concentration that had no significant inhibitory effect on keratinocyte metabolism, as determined by the MTT assay (data not shown). Induction of ICAM-1, HLA-DR, and β2-microglobulin on keratinocytes was not significantly affected by incubation with

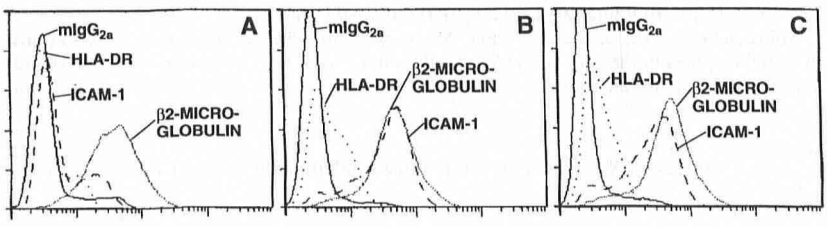


**Figure 1. Lipofectin does not significantly inhibit IFN-γ–induced upregulation of keratinocyte ICAM-1, HLA-DR, or β2-microglobulin expression.** Untreated or IFN-γ–treated keratinocytes were trypsinized prior to staining with $mIgG_{2a}$ or MoAb against HLA-DR, ICAM-1, or β2-microglobulin (*A–C*). Keratinocytes do not constitutively express ICAM-1 and HLA-DR but do express significant amounts of β2-microglobulin (*A*). ICAM-1 and HLA-DR expression are significantly upregulated 24 h after stimulation with 1000 U/ml of IFN-γ (*B*). This IFN-γ–induced induction of ICAM-1 and HLA-DR and the constitutive expression of β2-microglobulin is not significantly inhibited when keratinocytes are cultured in media supplemented with 10 μg/ml lipofectin (*C*).

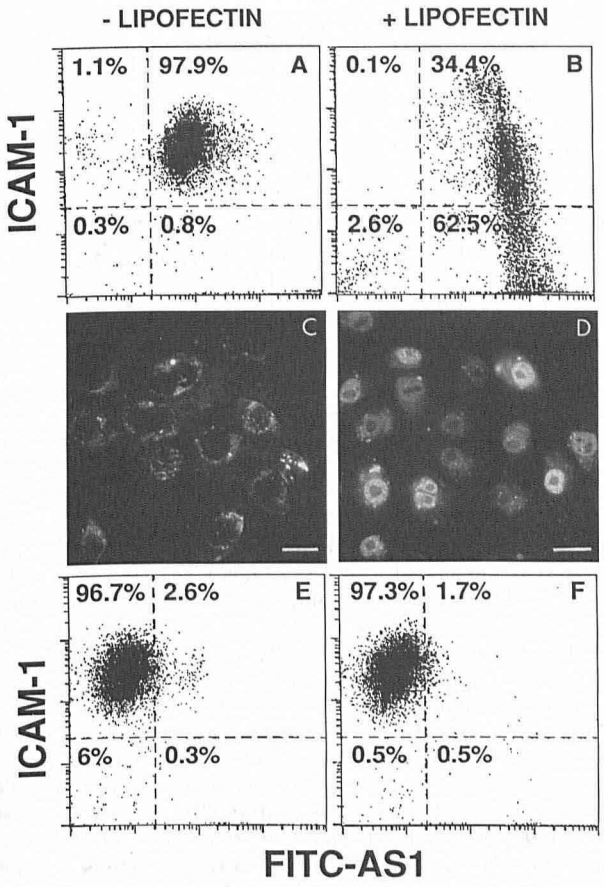


**Figure 2. The lipofectin-dependent nuclear distribution of ICAM-1 AS1 (S-ODN) is critical for the inhibition of IFN-γ–induced ICAM-1 expression.** Keratinocytes were treated with 0.5 μM of AS1 as a S-ODN (*A–D*) or PD-ODN (*E–F*) internally labeled with fluorescein (FITC). AS1 was either applied in the absence (*A,C,E*) or presence (*B,D,F*) of 10 μg/ml lipofectin. Cultures *C–D* were air-dried 24 h after treatment with AS1 (S-ODN) with (*C*) and without (*D*) lipofectin and mounted for fluorescence microscopy (*Scale bars*, 20 μm). Keratinocytes were also treated with FITC-labeled AS1 as a PD-ODN in the absence (*E*) or presence (*F*) of lipofectin. Cultures *A–B* and *E–F* were treated with IFN-γ after 24 h and trypsinized after 48 h. Cell suspensions were examined for fluorescence intensity either immediately or after incubation with a phycoerythrin (PE)–labeled MoAb against ICAM-1. Keratinocytes treated with FITC-AS1 as a S-ODN without lipofectin show a significant uptake of the antisense that has no effect on IFN-γ–induced expression of ICAM-1 (*A*). As shown in *C*, AS1 (S-ODN) accumulate in the cytoplasm. In contrast, AS1 (S-ODN) is taken up to a larger extent in the presence of lipofectin accompanied by a significant (>60%) inhibition of IFN-γ–induced ICAM-1 expression (*B*). Under these conditions, FITC-AS1 is primarily localized throughout the cell (*D*). When FITC-AS1 is applied as a PD-ODN in the presence (*E*) or absence (*F*) of 10 μg/ml lipofectin, there is only marginal green fluorescence with no alteration of ICAM-1 expression (*E*).

lipofectin when compared to control cultures not receiving lipofectin **(Fig 1)**.

**Lipofectin-Mediated Nuclear Uptake of ICAM-1 Antisense S-ODN Correlates with Biologic Activity** Keratinocytes treated with a fluorescinated (FITC) phosphorothioate (S-ODN) ICAM-1 antisense (AS1) in the absence of lipofectin accumulated FITC-AS1 **(Fig 2*A*)**, which showed a cytoplasmic distribution **(Fig 2*C*)**. Cytoplasmic uptake of AS1 S-ODN did not alter IFN-γ–induced ICAM-1 expression **(Fig 2*A*)**. In contrast, treatment of keratinocytes with FITC-AS1 (S-ODN) plus lipofectin resulted in an increased cellular uptake **(Fig 2*B*)** with prominent localization in the nucleus **(Fig 2*D*)** and a significant decrease in the staining intensity for ICAM-1 **(Fig 2*B*)**. FITC-labeled PD-AS1 did not accumulate in cells in the absence **(Fig 2*E*)** or presence **(Fig 2*F*)** of lipofectin.

**Differential Inhibition of IFN-γ–Induced ICAM-1 Expression on Keratinocytes by S-ODN AS1 and AS2** The ability of most keratinocytes to express ICAM-1 after IFN-γ treatment was inhibited by AS1 (which targets a site in the 3′-untranslated region of the ICAM-1 mRNA) when keratinocytes were used at the fourth to sixth passages. Inhibition of ICAM-1 expression on up to 70% of keratinocytes was achieved at a concentration of 0.5 μM **(Fig 3*B*)**. Two scrambled S-ODN (SCR1a and SCR1b) with a nucleotide composition identical to that of AS1 also significantly inhibited ICAM-1 expression on keratinocytes but to a lesser extent **(Fig 3*C–D*)**. However, no inhibition of ICAM-1 expression was seen in the presence of a degenerate 21-mer antisense (DEG AS) that had a random nucleotide composition at each position **(Fig 3*E*)**. When a phosphodiester analog of AS1, PD-AS1, was used in the presence of lipofectin at 0.5 to 100 μM, neither IFN-γ–induced ICAM-1 nor HLA-DR expression on keratinocytes were affected (data not shown). Fluorescence-activated cell sorter (FACS) analysis of keratinocytes treated with a FITC-labeled analog of PD-AS1 revealed that only minute amounts of PD-AS1 could be detected in keratinocytes **(Fig 2*E,F*)**. Immunofluorescence microscopy of keratinocytes treated with FITC–PD-AS1 did not reveal significant intracellular fluorescence (data not shown). AS2, the S-ODN that targets the AUG initiation codon of the ICAM-1 mRNA, had no inhibitory effect on either IFN-γ–induced ICAM-1 or HLA-DR expression at concentrations of 0.2 to 20.0 μM (data not shown).

**Specificity of Inhibition of ICAM-1 AS1** To determine whether AS1 specifically targeted ICAM-1 expression, the effect of AS1 on HLA-DR, β2-microglobulin, and α3- and β1-integrin expression was also studied **(Fig 4)**. IFN-γ–induced HLA-DR expression on keratinocytes was significantly, although not completely reduced with AS1 **(Fig 4*A*)**. Similar inhibition of HLA-DR expression was seen in the presence of the controls SCR1a and SCR1b **(Fig 4*B–C*)**, but not by the DEG AS (data not shown). Because HLA-DR is expressed only at moderate levels 24 h after treatment with IFN-γ, the effect of the S-ODN on IFN-γ–induced HLA-DR expression was also studied after 48 h. Even though HLA-DR was upregulated to a greater extent at this time, it was significantly inhibited by AS1 and the scrambled controls SCR1a and SCR1b (data not shown). To determine whether this inhibition was restricted to IFN-γ–induced molecules, other surface molecules were studied. β2-microglobulin is constitutively expressed on keratinocytes but is also upregulated to some extent upon treatment with IFN-γ **(Fig 1)**. Neither AS1 nor the scrambled controls SCR1a and SCR1b significantly inhibited β2-microglobulin expression on keratinocytes **(Fig 4*D–F*)**. In addition, constitutive expression of α3- and β1-integrins was not affected by AS1 or SCR1a **(Fig 4*G–J*)**.

**Inhibition of ICAM-1 Expression by AS1 Is Prevented by Preincubation with the Complementary Phosphorothioate Sense Strand** To determine whether the observed inhibition of ICAM-1 and HLA-DR by ICAM-1 AS1 could be prevented, AS1 was preincubated with the complementary sense strand SS1 identical to the targeted mRNA region prior to treatment of keratinocytes **(Fig 5)**. The sense strand SS1 alone at 0.2–1.0 μM had no inhibitory effect on IFN-γ–induced ICAM-1 or HLA-DR expression on keratinocytes (data not shown). Preincubation of AS1 with equal concentrations of SS1 abrogated antisense-dependent inhibition of ICAM-1 and HLA-DR expression. In contrast, preincubation of AS1 with the degenerate 21-mer (DEG AS) had no significant effect on ICAM-1 and HLA-DR inhibition **(Fig 5)**. Preincubation of SCR1a and SCR1b with SS1 partially prevented their inhibition of ICAM-1 and HLA-DR expression whereas preincubation with the DEG AS did not (data not shown).

**Inhibition of IFN-γ–induced ICAM-1 Expression by AS1 Is Prevented by Preincubation with the Phosphorothioate**

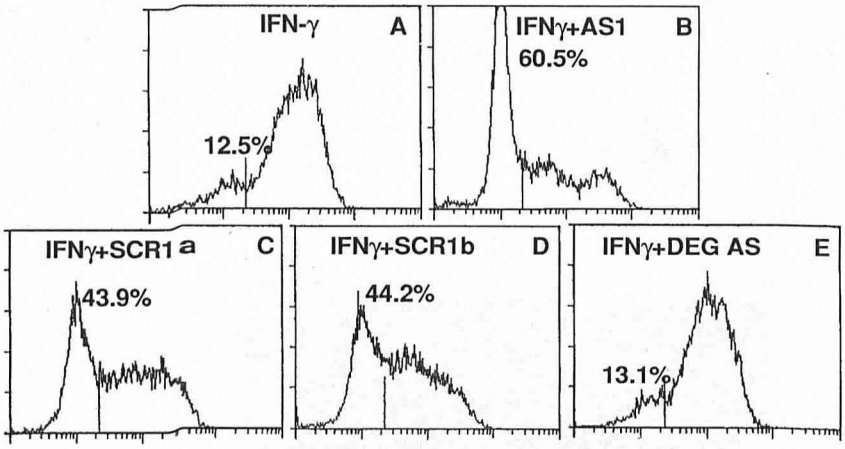


**Figure 3. ICAM-1 AS1 inhibits ICAM-1 expression on keratinocytes.** Keratinocytes were treated with AS1, SCR1a, SCR1b, and DEG AS in the presence of 10 μg/ml lipofectin for 4 h and were stimulated with 1000 &/ml IFN-γ for an additional 24 h. Cell suspensions were then reacted with a MoAb against ICAM-1. Greater than 85% of the keratinocytes are ICAM-1+ after treatment with IFN-γ (*A*) as compared to 0–5% in untreated keratinocytes (gates are set on untreated keratinocytes reacted with a MoAb against ICAM-1). AS1 at 0.5 μM inhibits ICAM-1 on 60.5% of all keratinocytes (*B*). Two scrambled controls for AS1 with identical nucleotide compositions, SCR1a (*C*) and SCR1b (*D*), also significantly inhibit ICAM-1 expression at a concentration of 0.5 μM, though to a lesser extent. No inhibition of ICAM-1 expression is seen in the presence of a degenerate 21-mer S-ODN, DEG AS, at 0.5 μM (*E*).

**Trinucleotide G-G-G** Because AS1 displays significant homology with SCR1a and SCR1b due to the high content of cytosines **(Table I)**, we wanted to further study the relevance of shared nucleotide sequences in the inhibition of IFN-γ–induced ICAM-1 and HLA-DR expression. Phosphorothioate trinucleotides complementary to common nucleotide repeats in AS1, SCR1a, and SCR1b were preincubated with AS1 and SCR1a to test their ability to block S-ODN–dependent inhibition of the IFN-γ–induced expression of ICAM-1 and HLA-DR. These S-ODN were G-G-G, G-A-G, T-G-G, G-T-G, and G-G-A. Preincubation of 0.5 μM AS1 with G-G-G at 5 μM completely prevented AS1-dependent inhibition of IFN-γ–induced ICAM-1 expression **(Fig 6)** as well as HLA-DR expression (data not shown). Preincubation of SCR1a with G-G-G abrogated inhibition of IFN-γ–induced ICAM-1 or HLA-DR expression by SCR1a in an analogous manner (data not shown). Coculture of keratinocytes with G-G-G at 5 μM alone did not alter IFN-γ–induced expression of ICAM-1 or HLA-DR. In contrast, preincubation of AS1 at 0.5 μM with either S-ODN T-G-G, G-T-G, G-G-A, or G-A-G **(Fig 6)** at 5 μM did not affect inhibition of IFN-γ–induced ICAM-1 or HLA-DR expression by AS1. In one experiment, S-ODN of different length (3- to 21-mer) consisting only of cytosines neither inhibited IFN-γ–induced ICAM-1 nor HLA-DR expression at 5 μM (3-mer) or 0.5 μM (6-, 9-, 15-, 21-mer) (data not shown).

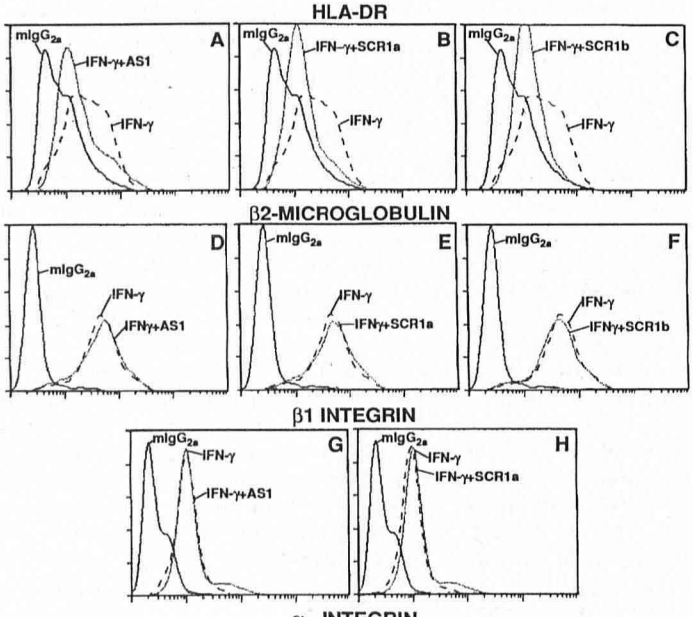


**Figure 4. ICAM-1 AS1 partially inhibits expression of HLA-DR but not of β2-microglobulin, β1-integrin, and α3-integrin.** Keratinocytes were treated with AS1, SCR1a, or SCR1b at 0.5 μM plus lipofectin for 4 h prior to stimulation with IFN-γ for an additional 24 h. Cells were then trypsinized and some cell suspensions were reacted with $mIgG_{2a}$ or MoAb against HLA-DR (*A–C*), β2-microglobulin (*D–F*), β1-integrin (*G–H*), or $mIgG_1$ plus MoAb against α3-integrin (*I–J*). IFN-γ–induced HLA-DR expression on keratinocytes is significantly inhibited by AS1 (*A*), SCR1a (*B*), and SCR1b (*C*). Expression of β2-microglobulin is not inhibited by AS1 (*D*), SCR1a (*E*), and SCR1b (*F*). Constitutive expression of β1-integrin is not altered by AS1 (*G*) or SCR1a (*H*). α3-integrin expression is also not affected by AS1 (*I*) or SCR1a (*J*).

## DISCUSSION

We have studied the effects of two ICAM-1 phosphorothioate oligodeoxynucleotides (S-ODN) on IFN-γ–induced expression of

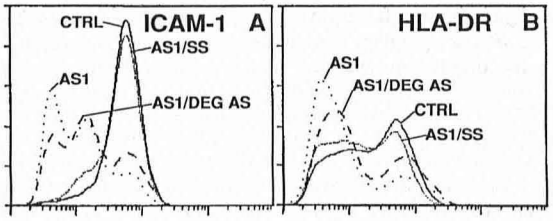


**Figure 5. Sequence-specific inhibition of IFN-γ–induced ICAM-1 or HLA-DR expression by AS1 is blocked by the complementary sense strand.** Keratinocytes were treated with AS1 alone, AS1 plus SS, or AS1 plus DEG AS (all at 0.5 μM) prior to stimulation with IFN-γ. Cell suspensions were then stained with a MoAb against ICAM-1 (*A*) or HLA-DR (*B*). Most keratinocytes express ICAM-1 (*A*) upon treatment with IFN-γ (CTRL, ——). Incubation of keratinocytes with AS1 at 0.5 μM prior to stimulation with IFN-γ significantly inhibits ICAM-1 expression (. . .). When AS1 is preincubated with equal concentrations of SS, antisense-dependent inhibition of ICAM-1 is completely abrogated (.....). Preincubation of AS1 with DEG AS does not block inhibition of ICAM-1 expression (– – –). HLA-DR expression (*B*) is similarly induced by IFN-γ after 48 h on most keratinocytes (CTRL, ——). Incubation of keratinocytes with AS1 at 0.5 μM prior to stimulation with IFN-γ significantly inhibits HLA-DR expression (. . .). When AS1 is preincubated with equal concentrations of SS, antisense-dependent inhibition of HLA-DR is completely abrogated (.....). Preincubation of AS1 with DEG AS does not block inhibition of HLA-DR expression (– – –).

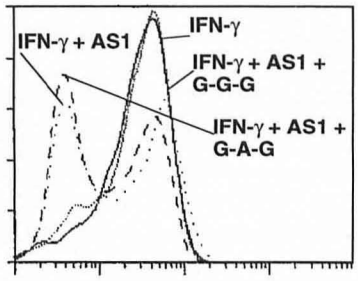


**Figure 6. Phosphorothioate trinucleotides complementary to repeated nucleotide sequences in AS1 have differential effects on the inhibition of IFN-γ–induced ICAM-1 expression by AS1.** Keratinocytes were treated with AS1 at 0.5 μM alone or with AS1 at 0.5 μM preincubated with the trinucleotides G-G-G or G-A-G (all at 5.0 μM) prior to stimulation with IFN-γ. IFN-γ–stimulated keratinocytes and cells treated as described above were then stained with a MoAb against ICAM-1. Most IFN-γ–treated keratinocytes express ICAM-1 (——). Treatment with AS1 significantly inhibits IFN-γ–induced ICAM-1 expression (. . .). Preincubation of AS1 with the S-ODN G-G-G completely blocks this inhibition of ICAM-1 expression (....). In contrast, preincubation of AS1 with the S-ODN G-A-G does not prevent AS1-dependent inhibition of IFN-γ–induced ICAM-1 expression (– – –).

ICAM-1 on human keratinocytes. These two S-ODN, targeting the AUG translation initiation codon (AS2) and a stem-loop structure at the 3′-untranslated region (AS1), have been previously identified as efficient inhibitors of cytokine (IFN-γ, interleukin-1, tumor necrosis factor-α)–induced ICAM-1 expression on endothelial cells and a lung carcinoma cell line [16]. In contrast to these recently reported findings [16], only AS1, targeting the 3′-untranslated region of the ICAM-1 mRNA, proved to be active in inhibiting the IFN-γ–induced upregulation of ICAM-1 expression on human keratinocytes. AS2, targeting the AUG initiation codon region did not inhibit ICAM-1 on keratinocytes even at tenfold higher concentrations than had been reported to be efficient [16].

As demonstrated by Chiang *et al*, significant inhibition of IFN-γ–induced ICAM-1 expression was only achieved when antisense S-ODN were complexed to the cationic lipid lipofectin [16]. In our experiments, lipofectin at 10 μg/ml significantly increased the uptake of antisense S-ODN **(Fig 2)**. Perhaps more importantly, lipofectin altered the subcellular distribution of the S-ODN, as previously described [5,8]: lipofectin-dependent nuclear enrichment of AS1 (S-ODN) correlated directly with the inhibition of IFN-γ–induced ICAM-1 expression by AS1 **(Fig 2)**. Lipofectin alone did not significantly alter IFN-γ–induced upregulation of ICAM-1 and HLA-DR expression and the cellular metabolism of keratinocytes **(Fig 1)** [16]. Our results strongly suggest a critical role for the nuclear localization of the S-ODN in antisense inhibition. However, these findings are in contrast to those of Nestle *et al*, who demonstrated a significant nuclear uptake of S-ODN by up to 38% keratinocytes in the absence of lipofectin [19]. Their system differed from ours in that keratinocytes were treated with S-ODN at tenfold higher concentrations. No explanation was given in their study why only a minority of keratinocytes showed this particular pattern of intracellular uptake. However, both studies demonstrated that there is a significant increase in the intracellular uptake of S-ODN in the presence of lipofectin in essentially all keratinocytes with a typical nuclear staining pattern that goes along with the biologic activity of the AS S-ODN.

These studies also illustrate the importance of choosing appropriate controls to rule out non–antisense-specific effects caused by the nucleotide composition of a particular antisense reagent. We designed two scrambled S-ODN controls with a nucleotide composition identical to ICAM-1 AS1. Both of these caused a significant, although less pronounced (than AS1), inhibition of ICAM-1 expression **(Fig 3)**. SCR1a and SCR1b also inhibited HLA-DR expression to some extent. To control for effects of the phosphorothioate backbone, a degenerate 21-mer S-ODN (DEG AS) was also used as a control and had no significant inhibitory effects on ICAM-1 expression. We thus concluded that these scrambled control S-ODN displayed intrinsic pharmacologic effects dependent on their nucleotide composition **(Table I)**. In this particular case, the high content of cytosines in AS1 did not allow a design of a scrambled ODN without partial homology to AS1, which may allow some degree of hybridization to the site of the ICAM-1 mRNA complementary to AS1 **(Table I)**. The observed inhibition of ICAM-1 by AS1 was sequence-specific and required single-stranded ODN in that preincubation of AS1 with a "sense strand" S-ODN identical to the targeted mRNA region (SS1) abolished the inhibitory effect of AS1 on ICAM-1 expression **(Fig 5)**.

Preincubation experiments with trinucleotides complementary to nucleotide sequences shared by AS1 and the control S-ODN SCR1a and SCR1b demonstrated that the cytosine repeats (C-C-C) contributed to the observed inhibition of IFN-γ–induced ICAM-1 and HLA-DR expression by all S-ODN. The significance of this finding is, however, unknown. In the case of AS1, hybridization of the G-G-G S-ODN to the complementary cytosine triplet (5×) in the antisense may prevent hybridization to the targeted (complementary) mRNA region. Alternatively, aptameric inhibition of ICAM-1 and HLA-DR expression, depending on the C-C-C triplet in AS1, SCR1a, and SCr1b, could also be inhibited by the G-G-G sequence. Evidence for a non–sequence specific inhibition of a gene product based on the nucleotide content of the antisense has been also presented by Yawsen *et al* [7]. In that study, the expression of the NB-1 gene (which encodes for a calmodulin-like protein) could be inhibited by antisense ODN containing contiguous stretches of guanosine residues that were not complementary to regions in the NB-1 mRNA. However, our findings with the cytosine-only S-ODN that showed no inhibitory properties would mitigate against this possibility.

The phosphorothioate backbone structure was critical for ICAM-1 AS1 to be effective, as an identical ODN with a phosphodiester backbone had no significant inhibitory effect on ICAM-1 expression at concentrations ranging from 0.5 to 100.0 μM. This lack of inhibition is most likely due to the intracellular degradation of the PD-ODN by nucleases. Evidence for this has been presented in that keratinocytes treated with FITC-labeled PD-AS1 show minimal uptake for PD-AS1 (as determined by FACS analysis and immunofluorescence microscopy) in contrast to the highly nuclease-resistant S-ODN analog, which shows a five- to tenfold higher intracellular accumulation **(Fig 2)**.

Because IFN-γ–induced HLA-DR expression was inhibited by AS1 to some extent, additional surface molecules, such as β2-microglobulin, β1-integrin, and α3-integrin were studied. β2-microglobulin expression was not affected by AS1, which is consistent with the findings of Chiang *et al* [16]. The same observation was made for α3- and β1-integrins, which are constitutively expressed on keratinocytes. We considered the possibility that HLA-DR expression was inhibited non-specifically because it was only minimally upregulated after 24 h. This was not the case, however, because HLA-DR expression was also significantly inhibited 48 h after treatment of keratinocytes with IFN-γ. Sequence-specific inhibition was unlikely because the mRNA for HLA-DR contains no regions complementary to AS1. However, the mRNAs for the different HLA-DR alleles contain guanosine repeats (G-G-G) at a relatively high frequency, which may account for a partial hybridization of the cytosine-rich AS1, SCR1a, and SCR1b. This may explain why the scrambled S-ODN controls SCR1a and SCR1b also inhibited HLA-DR expression.

Inhibition of IFN-γ–induced ICAM-1 and HLA-DR expression by AS1 through an interaction with an IFN-γ–responsive regulatory gene region or protein seems rather unlikely because IFN-γ–induced upregulation of $\beta_2$-microglobulin was not affected. Furthermore, AS1 also inhibited TNF-α– and IL-1β–induced ICAM-1 expression [16]. The antisense non-specific inhibition of IFN-γ–induced HLA-DR expression by ICAM-1 AS1 in our study is in contrast to the findings of Nestle *et al* [19]. In their study, an AS

S-ODN targeting a site in the 3′-untranslated region of the ICAM-1 mRNA inhibited IFN-γ–induced expression of ICAM-1 but not of HLA-DR. The conditions of their study were different in that the ICAM-1 AS S-ODN (with a different nucleotide composition) was used at 5 μM (instead of 0.5 μM in our study), lipofectin at 5 μg/ml (instead of 10 μg/ml), and IFN-γ at 10 U/ml for 48 h (instead of 1000 U/ml for 24 h in our study). The lack of comparability of both experimental systems is reflected by their finding that, at 1000 U/ml of IFN-γ (used in our study), there was no significant inhibition of IFN-γ–induced ICAM-1 expression with their AS S-ODN.

In addition to antisense-specific effects, antisense–non-specific activities seem to be a common effect of some S-ODN. Bennett *et al* found a significant inhibition of TNF-α–induced expression of vascular cell adhesion molecule (VCAM) in human umbilical vein endothelial cells upon treatment with antisense S-ODNs against E-selection and ICAM-1, or even with a control S-ODN [20]. The authors concluded that these E-selectin and ICAM-1 S-ODN acted selectively on the targeted adhesion molecule in that VCAM expression was not affected at lower concentrations of these S-ODN, although these concentrations were still effective in inhibiting the targeted gene product [20]. The same observation holds true for the HLA-DR inhibition by AS1 in our studies. There is virtually no inhibition at very low concentrations (0.1 μM)—the same concentration that causes some inhibition of IFN-γ–induced ICAM-1 expression.

In summary, this study shows the complex biologic effects exerted by antisense ODN in addition to their antisense-specific effects on the targeted gene product. We have addressed some factors (use of lipids for the quantitative and qualitative delivery of ODN, backbone structure, controls, etc.) that are critical for the use of antisense ODN in human keratinocytes. Only careful consideration of these factors will allow one to take advantage of the potential of antisense ODN as modulators of gene expression *in vitro* and *in vivo*.

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## REFERENCES

1. Mirabelli CK, Bennett CF, Anderson K, Crooke ST: In vitro and in vivo pharmacologic activities of antisense oligonucleotides. *Anti-Cancer Drug Design* 6:647–655, 1991
2. Marshall WS, Caruthers MH: Phosphorodithioate DNA as a potential therapeutic drug. *Science* 259:1564–1569, 1993
3. Stein CA, Cheng YC: Antisense oligonucleotides as therapeutic agents—is the bullet really magic? *Science* 261:1004–1012, 1993
4. Distefano MD, Dervan BP: Energetics of cooperative binding of oligonucleotides with discrete dimerization domains to DNA by triple helix formation. *Proc Natl Acad Sci USA* 90:1179–1183, 1993
5. Wagner RW, Matteucci MD, Lewis JG, Gutierrez AJ, Moulds C, Froehler BC: Antisense gene inhibition by oligonucleotides containing c-5 propyne pyrimidines. *Science* 260:1510–1519, 1993
6. Neckers LM: Cellular internalization of oligodeoxy-nucleotides. In: Crooke ST, Lebleu B (eds.). *Antisense Research and Applications*. CRC Press, Boca Raton, 1993, pp 451–460
7. Yaswen P, Stampfer MR, Ghosh K, Cohen JS: Effects of sequence of thioated oligonucleotides on cultured human mammary epithelial cells. *Antisense Res Develop* 3:67–77, 1993
8. Bennett CF, Chiang MY, Chan H, Shoemaker JE, Mirabelli CK: Cationic lipids enhance cellular uptake of phosphorothioate antisense oligonucleotides. *Mol Pharmacol* 41:1023–1033, 1992
9. Matsukura M, Zon G, Shinozuka K, Robert-Guroff M, Shimada T, Stein CA, Mitsuya H, Wong-Staal F, Cohen JS, Broder S: Regulation of viral expression of human immunodeficiency virus in vitro by an antisense phosphorothioate oligodeoxynucleotide against rev (art/trs) in chronically infected cells. *Proc Natl Acad Sci USA* 86:4244–4248, 1989
10. Neckers L, Whitesell L, Rosolen A, Geselowitz DA: Antisense inhibition of oncogene expression. *Crit Rev Oncogenesis* 3:175–231, 1992
11. Biro S, Fu YM, Yu ZX, Epstein SE: Inhibitory effects of antisense oligodeoxynucleotides targeting c-myc mRNA on smooth muscle cell proliferation and migration. *Proc Natl Acad Sci USA* 90:654–658, 1993
12. Harel-Bellan A, Durum S, Muegge K, Abbas A, Farrar W: Specific inhibition of lymphokine. Biosynthesis and autocrine growth using antisense oligonucleotides in TH1 and TH2 helper T cell clones. *J Exp Med* 168:2309–2314, 1988
13. Maier JA, Voulalas P, Roeder D, Maciag T: Extension of the life span of human endothelial cells by an interleukin 1a antisense oligomer. *Science* 249:1570–1575, 1990
14. Benbernon N, Matsiota-Bernard P, Guenounou M: Antisense oligonucleotides to interleukin-4 regulate IgE and IgG2a production by spleen cells from Nippostromgylus brasiliensis-infected rats. *Eur J Immunol* 23:659–668, 1993
15. Dustin ML, Singer KH, Tuck DT, Springer TA: Adhesion of T lymphoblasts to epidermal keratinocytes is regulated by intercellular adhesion molecule 1 (ICAM-1). *J Exp Med* 167:1323–1331, 1988
16. Chiang MY, Chan H, Zounes MA, Freier SM, Lima WF, Bennett CF: Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms. *J Biol Chem* 266:18163–18174, 1991
17. Caughman SW, Lian-Jie L, Degitz K: Characterization and functional analysis of interferon-γ induced intercellular adhesion molecule-1 expression in human keratinocytes and A-431 cells. *J Invest Dermatol* 94:22S–26S, 1990
18. Noonberg SB, Garovoy MR, Hunt CA: Characteristics of oligonucleotide uptake in human keratinocyte cultures. *J Invest Dermatol* 101:727–731, 1993
19. Nestle FO, Mitra RS, Bennett CF, Chan H, Nickoloff BJ. Cationic lipid is not required for uptake and selective inhibitory activity of ICAM-1 phosphorothioate antisense oligonucleotides in keratinocytes. *J Invest Dermatol* 103:569–575, 1994
20. Bennett CF, Condon TP, Grimm S, Chan H, Chiang MY: Inhibition of endothelial cell adhesion molecule expression with antisense oligonucleotides. *J Immunol* 152:3530–3539, 1994